

Phototropins Promote Plant Growth in Response to Blue Light in Low Light Environments^W

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Phototropins (*phot1* and *phot2*) are plant-specific blue light receptors for phototropism, chloroplast movement, leaf expansion, and stomatal opening. All these responses are thought to optimize photosynthesis by helping to capture light energy efficiently, reduce photodamage, and acquire CO₂. However, experimental evidence for the promotion of plant growth through phototropins is lacking. Here, we report dramatic phototropin-dependent effects on plant growth. When plants of *Arabidopsis thaliana* wild type, the *phot1* and *phot2* mutants, and the *phot1 phot2* double mutant were grown under red light, no significant growth differences were observed. However, if a very low intensity of blue light (0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$) was superimposed on red light, large increases in fresh weight up to threefold were found in those plants that carried functional *PHOT1* genes. When the intensity of blue light was increased to 1 $\mu\text{mol m}^{-2} \text{s}^{-1}$, the growth enhancement was also found in the *phot1* single mutant, but not in the double mutant, indicating that *phot2* mediated similar responses as *phot1* with a lower sensitivity. The effects occurred under low photosynthetically active radiation in particular. The well-known physiological phototropin-mediated responses, including chloroplast movement, stomatal opening, and leaf expansion, in the different lines tested indicated an involvement of these responses in the blue light-induced growth enhancement. We conclude that phototropins promote plant growth by controlling and integrating a variety of responses that optimize photosynthetic performance under low photosynthetically active radiation in the natural environment.

INTRODUCTION

Efficient use of solar energy for photosynthesis is important for plant growth and survival, especially in low light environments. Plants use light not only as an energy source for photosynthesis but also as an environmental signal and respond to its intensity, wavelength, and direction. Light is perceived by plant photoreceptors such as phytochromes, cryptochromes, and phototropins, and plants generate a wide range of specific physiological responses through these receptors.

In *Arabidopsis thaliana*, at least 10 photoreceptors, including five phytochromes (*phyA* through *phyE*), three cryptochromes (*cry1*, *cry2*, and *cry3*), and two phototropins (*phot1* and *phot2*), have been identified (Arabidopsis Genome Initiative, 2000). Phytochromes that absorb red/far-red light and cryptochromes that sense UV-A/blue light coordinately regulate photomorphogenic processes, including deetiolation, vegetative growth, flowering induction, and circadian rhythms (Smith, 2000; Lin, 2002; Morelli and Ruberti, 2002; Wang and Deng, 2002). Phytochrome also regulates seed germination and shade avoidance. By contrast, phototropins that absorb UV-A/blue light have been

suggested to play an important role in photo-induced movement responses (Briggs and Christie, 2002).

A phototropin was first cloned as a blue light receptor responsible for phototropic bending, using an *Arabidopsis* mutant impaired in phototropism (Huala et al., 1997). The mutants lacked light-dependent phosphorylation of a 120-kD protein that appeared related to phototropism. The action spectrum of in vivo phosphorylation of this protein and the fluence dependency of the phosphorylation were similar to those of physiological phototropic responses. The cloned gene, initially named *NPH1* (for *NONPHOTOTROPIC HYPOCOTYL 1*), contained two repeated motifs assigned as LOV (Light, Oxygen, Voltage) domains in the N terminus and a Ser/Thr protein kinase domain in the C terminus (Huala et al., 1997; Briggs and Christie, 2002). The gene later was renamed *PHOT1*. Thereafter, a *PHOT1* homolog (*NPH1-like 1*) has been cloned from *Arabidopsis* and was renamed *PHOT2* (Jarillo et al., 1998; Briggs and Christie, 2002). *phot1* underwent autophosphorylation in response to blue light in insect cells and was demonstrated to be an autophosphorylating receptor kinase (Christie et al., 1998, 1999).

Using an *Arabidopsis* mutant impaired in chloroplast movement, *phot2* has been demonstrated to be responsible for the strong-light avoidance response (Jarillo et al., 2001; Kagawa et al., 2001; Kasahara et al., 2002). Moreover, *phot2* also is responsible for phototropic curvature in response to relatively high intensities of blue light (Sakai et al., 2001). Using the *Arabidopsis phot1 phot2* double mutant, it has been demonstrated that *phot1* and *phot2* redundantly mediate stomatal opening; the blue light-dependent H⁺-pumping activity that drives stomatal opening is lost in the guard cells of mutant plants (Kinoshita et al., 2001; Doi et al., 2004). More recently, both *phot1*

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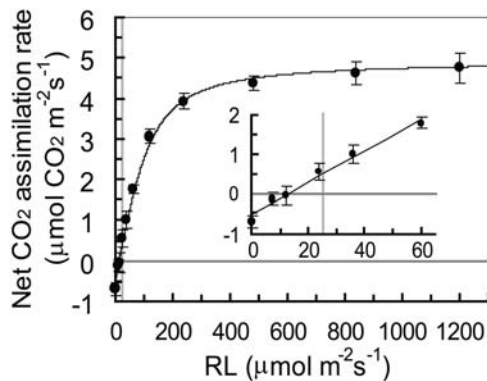


Figure 1. Light-Response Curve of Net CO₂ Assimilation Rate in Leaves of the Wild Type.

Red light (RL) intensity (equivalent to PAR) varied from 7.2 to 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Each point represents the mean of three measurements \pm SE. Vertical gray line indicates the light intensity used in growth tests ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$).

and *phot2* have been suggested to mediate leaf expansion (Sakamoto and Briggs, 2002), and *phot1* has been implied to be involved in the rapid inhibition of hypocotyl growth (Folta and Spalding, 2001). In contrast with this growth inhibition that is specifically mediated by *phot1*, genetic studies have revealed that *phot1* and *phot2* have partially overlapping functions in mediating phototropism, chloroplast movements, stomatal opening, and leaf expansion (Kagawa et al., 2001; Kinoshita et al., 2001; Sakai et al., 2001; Briggs and Christie, 2002). *phot1* seems to be more sensitive to blue light than *phot2* in triggering these responses, although sufficient documentation is yet to be provided for leaf expansion.

The blue light-induced responses mediated by phototropins optimize photosynthesis by improving the efficiency of light capture, reducing photodamage, and regulating the gas exchange between leaves and atmosphere. If so, we might expect to find an enhancement of plant growth in response to blue light through the stimulation of photosynthesis. However, no experimental evidence for the promotion of plant growth through phototropins is available to date.

In this study, we provide evidence that phototropin-mediated responses elicit a significant increase in plant growth, particularly under low photosynthetically active radiation (PAR). We also demonstrate that both *phot1* and *phot2* mediate this response and that the *phot1*-dependent pathway is more sensitive than the *phot2*-dependent one.

RESULTS

Phototropins Mediate Plant Growth under Light-Limited Conditions in Response to Blue Light

It is possible that blue light enhances plant growth if phototropins optimize photosynthesis. To test this hypothesis, we initially determined the light-response curve of photosynthetic CO₂ fixation in Arabidopsis leaves under red light. We found the

light compensation point at $12.8 \mu\text{mol m}^{-2} \text{s}^{-1}$ and a half-saturation intensity of $75.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 1). When Arabidopsis seedlings of the wild type were grown under light-limited conditions for CO₂ fixation (i.e., at $25 \pm 4 \mu\text{mol m}^{-2} \text{s}^{-1}$ red light) for 4 weeks (Figure 1, gray line), the fresh weight of the green tissue was 27.1 ± 2.4 mg per plant (Figure 2A). If blue light at very low intensity (defined here as $0.1 \pm 0.01 \mu\text{mol m}^{-2} \text{s}^{-1}$) was superimposed on red light during the growth period, the green tissue fresh weight was increased threefold to 75.1 ± 4.0 mg per plant (Figure 2A). Figure 2B shows a typical case of 6-week-old plants grown under red light and red light supplemented with very low intensity of blue light, respectively. Because the blue light intensity did not amount to $>0.4\%$ of PAR in these experiments, blue light must have acted as a signal triggering the response.

We attempted to identify blue light receptors responsible for this response using photoreceptor mutants of Arabidopsis. As in the wild type, the blue light-induced growth enhancement was found in *phyA phyB* (*phyA-201 phyB-5*) as well as *cry1 cry2* (*hy4-3 cry2-1*) double mutants, but not in the *phot1 phot2* (*phot1-5 phot2-1*) double mutant (Figure 2A). However, growth of the *phyA phyB* double mutant was very slow, probably because of the disturbance of seed germination and deetiolation (Wang

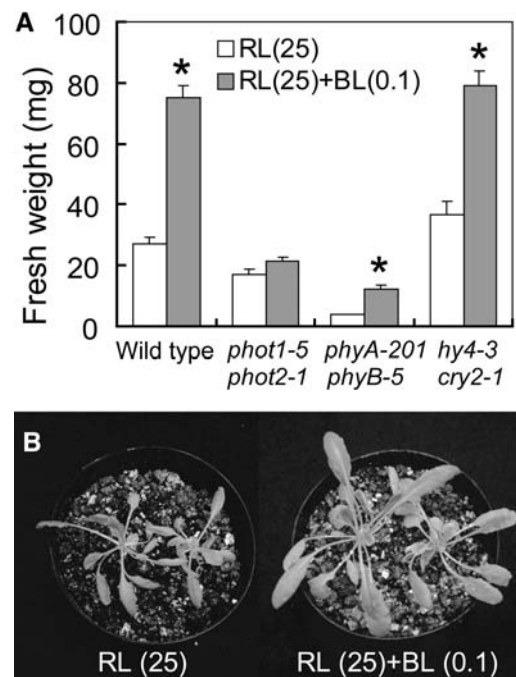


Figure 2. Blue Light-Induced Enhancement of Plant Growth under Low PAR of Red Light.

(A) Fresh weight of green tissues of wild-type and mutant plants. Wild-type, *phot1-5 phot2-1*, *phyA-201 phyB-5*, and *hy4-3 cry2-1* plants were grown for 4 weeks under red light (RL) ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) with or without blue light (BL) ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$). Bars represent means \pm SE ($n = 24$). Asterisks indicate significant differences in fresh weights between red light and red light with blue light ($P < 0.01$).

(B) Wild-type plants were grown for 5 to 6 weeks under red light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) with or without blue light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$).

and Deng, 2002). These results suggested that the growth enhancement by blue light is mediated by phot1 and/or phot2, but not by other photoreceptors.

Both phot1 and phot2 Mediate Blue Light-Induced Growth Enhancement

We further characterized the significance of phototropins for the blue light-induced growth enhancement using phototropin single (*phot1-5* and *phot2-1*) and double (*phot1-5 phot2-1*) mutants. When wild-type and mutant plants were grown for 4 weeks under light-limited red light conditions, no significant differences were observed in their fresh weight (white bars in Figure 3A). When very low blue light as defined above was supplemented, growth was enhanced in the wild type and *phot2-1*, but not in the *phot1-5* and the *phot1-5 phot2-1* double mutant (gray bars in Figures 3A and 3B). Thus, phot1 was responsible for the growth enhancement, and phot2 did not seem to be involved in the response to very low blue light. Because it appeared possible that expression of the phot2 protein was specifically suppressed under our growth conditions, we confirmed that phot2 was expressed in both the wild type and *phot1-5* to similar extents (Figure 4). The phot1 protein was expressed in both the wild type and *phot2-1*.

Because phot2 is known to mediate phototropic responses, chloroplast movement, and stomatal opening at higher blue light intensities, we examined the effect of 1.0 instead of 0.1 $\mu\text{mol m}^{-2} \text{s}^{-1}$ blue light on plant growth. Under these conditions, *phot1-5* responded to blue light, whereas the *phot1-5 phot2-1* double mutant did not (Figure 3C). This result indicated that phot2 also mediates blue light-induced growth responses, but that the phot1-dependent pathway is more sensitive than the phot2-dependent one. We confirmed that the phot1 protein was neither expressed in the single mutant *phot1-5* nor in the double mutant *phot1-5 phot2-1* (Figure 4). Furthermore, we found that the expression of phot proteins was not affected in the absence of blue light (data not shown).

PHOT1 Restores Blue Light-Induced Growth Enhancement in the phot1 phot2 Double Mutant

We transformed the *phot1 phot2* double mutant with the 35S-controlled *PHOT1* gene and obtained three lines (A, B, and C), with the highest transcript levels of *PHOT1* in A (data not shown). The blue light-dependent growth response was restored in response to a very low intensity of blue light in all of the transgenic lines and was most prominent in line A, demonstrating

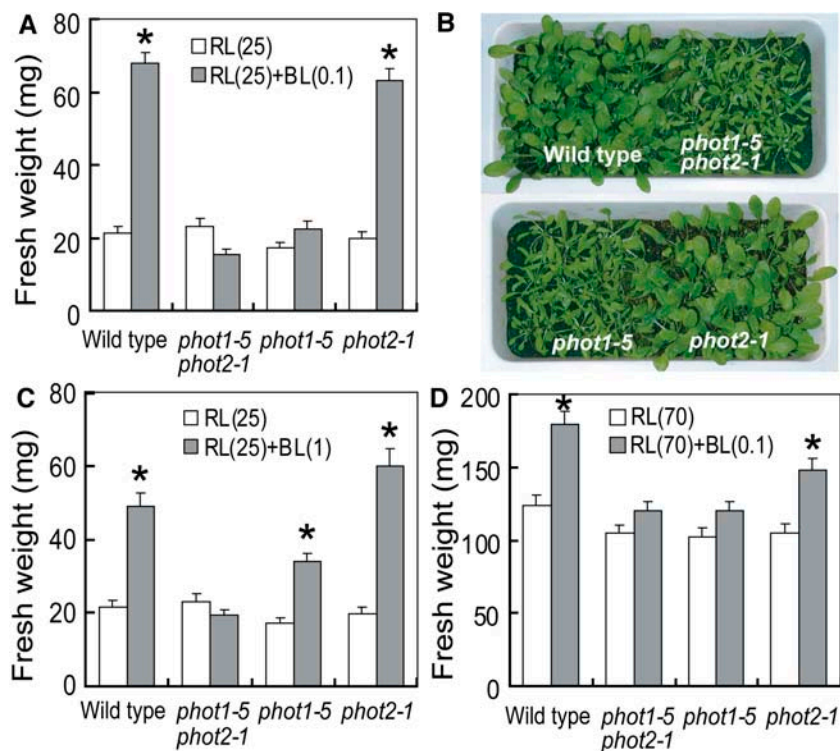


Figure 3. Phototropin-Mediated Growth Enhancement.

(A), (C), and (D) Fresh weight of green tissues of the wild type and mutants. Wild-type, *phot1-5*, *phot2-1*, and *phot1-5 phot2-1* plants were grown for 4 weeks under red light (RL) ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) with or without blue light (BL) ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) (A), red light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) with or without blue light ($1 \mu\text{mol m}^{-2} \text{s}^{-1}$) (C), and red light ($70 \mu\text{mol m}^{-2} \text{s}^{-1}$) with or without blue light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) (D). Bars represent means \pm SE ($n = 24$). Asterisks indicate significant differences in fresh weights between red light and red light with blue light ($P < 0.01$).

(B) Five- to six-week-old wild-type and mutant plants grown under the same conditions as in Figure 3A.

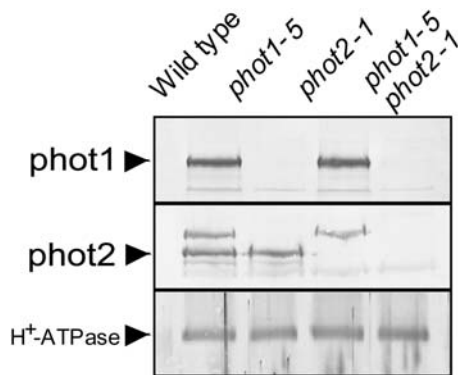


Figure 4. Expression of *phot1* and *phot2* Proteins Determined by Protein Blot Analysis.

Microsomal membranes were obtained from leaves of the wild type and mutants grown under the same conditions as in Figure 3A. Amounts of protein used were 50 μg for *phot1*, 80 μg for *phot2*, and 20 μg for H^+ -ATPase, respectively. The top band in the panel marked *phot2* is *phot1*.

unambiguously that *phot1* was responsible for the growth enhancement (Figure 5).

Chloroplast Accumulation, Stomatal Opening, and Leaf Expansion Mediated by Phototropins May Contribute to Growth Enhancement

To determine whether known phototropin-mediated physiological reactions correlate with the blue light-induced growth enhancement shown here, we measured chloroplast distribution and stomatal aperture in the wild type and mutants under our standard growth conditions. If very low blue light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$) was superimposed on red light, chloroplasts accumulated at the periclinal cell surfaces in both the wild type and *phot2*, but not in *phot1* and *phot1 phot2* (Figure 6A). Similarly, wider stomatal apertures were observed in the wild type and *phot2* than in *phot1* and *phot1 phot2* (Figure 6B). Chloroplast accumulation increases light absorption, and increased stomatal conductance enhances CO_2 uptake into the leaves; both factors optimize the efficiency of photosynthetic CO_2 fixation.

We measured the rate of photosynthetic CO_2 fixation under standard growth conditions to determine whether it actually was increased by the *phot1*-mediated responses. As shown in Figure 6C, photosynthetic CO_2 fixation rates in both wild-type and *phot2* mutant plants were higher than in *phot1* and *phot1 phot2* mutants. Essentially the same results were obtained when CO_2 fixation was expressed on the basis of chlorophyll content (data not shown). The enhanced rates of photosynthesis in the wild type and *phot2* corresponded to $\sim 5\%$ of the maximum rate under light-saturated conditions (Figure 1). The increased photosynthetic CO_2 fixation appeared correlated to the plant's capacity to show blue light-dependent growth responses (Figure 2A).

We also found that under standard growth conditions, leaves of *phot1* and *phot1 phot2* curled downward (Sakai et al., 2001; Sakamoto and Briggs, 2002), but those of the wild type and *phot2* did not (Figure 3B). We compared the surface area of artificially flattened leaves with the area of their projection in the undis-

turbed, more or less curled, state and found that the leaf area directly exposed to the light was reduced by $\sim 50\%$ because of curling in *phot1* and *phot1 phot2* (Figure 6D). Presumably, leaf curling decreases the photosynthetic efficiency by reducing the effective leaf area of light capturing, and this appears to contribute largely to the growth reduction of the mutants.

Phototropins Mediate Plant Growth under More Natural Light Conditions

As shown in previous reports, no obvious difference in green tissue growth was observed between wild-type plants and the *phot* mutant plants under the relatively high light intensities (Kasahara et al., 2002; Sakamoto and Briggs, 2002). Therefore, phototropins do not appear to improve light capture characteristics significantly under high light conditions. To see whether very low blue light affects growth under moderate PAR, we increased red light from 25 to $70 \mu\text{mol m}^{-2} \text{s}^{-1}$, a value close to the half-saturating intensity for Arabidopsis photosynthesis (Figure 1). In this environment, plants grew considerably faster than under low PAR conditions, but blue light still significantly enhanced green tissue fresh weight by 40% in both wild-type and *phot2* plants (Figure 3D).

Natural sunlight differs from the irradiation applied under our standard growth conditions in that its blue light portion is relatively larger than its red light fraction. To determine plant growth under more natural conditions, plants were cultivated under white light whose emission properties resembled those of sunlight. Under weak white light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$), in which PAR was the same as that of our standard growth conditions, the wild type, *phot1-5*, and *phot2-1* all grew considerably faster than *phot1-5 phot2-1* (Figures 7A and 7C). The growth difference between the *phot1-5* and *phot2-1* mutants was eliminated under this condition, suggesting that *phot2* functions to enhance plant growth under white light. If the intensity of white light was increased to $70 \mu\text{mol m}^{-2} \text{s}^{-1}$, the growth differences observed

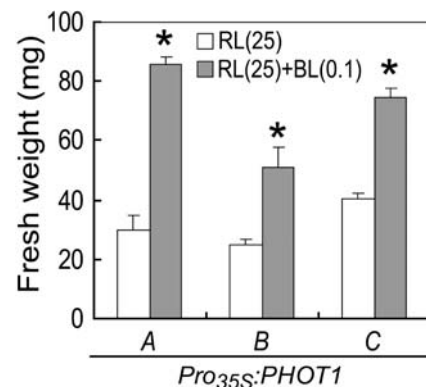


Figure 5. Complementation of Blue Light-Induced Growth Enhancement by Transformation of the *phot1 phot2* Double Mutant with *PHOT1*.

Fresh weight of green tissues of transgenic plants. Three independent lines (A, B, and C) of *phot1-5 phot2-1* transformed with the 35S-controlled *PHOT1* were grown for 4 weeks under the same conditions as in Figure 3A. BL, blue light; RL, red light.

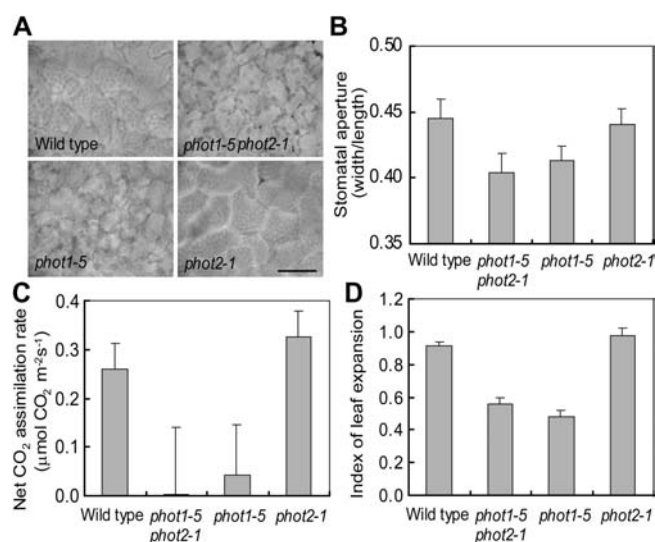


Figure 6. Phototropin-Mediated Physiological Responses under Red Light ($25 \mu\text{mol m}^{-2} \text{s}^{-1}$) with Blue Light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$).

(A) Distribution of chloroplasts in mesophyll cells of wild-type and mutants leaves. Micrographs were taken from the adaxial side of leaves. Bar = $50 \mu\text{m}$.

(B) Stomatal aperture in leaves of the wild type and mutants. Apertures are expressed as the ratio of width to length of the guard cell pair, as described in Methods. Bars represent means \pm SE ($n = 30$).

(C) Net CO₂ assimilation rate in leaves of the wild type and mutants. Bars represent means \pm SE ($n = 10$).

(D) Leaf expansion of the wild type and mutants. The degree of expansion is expressed as the ratio of the projection of the leaf before and after artificial uncurling. Bars represent means \pm SE ($n = 5$).

under low PAR were greatly reduced (Figures 7B and 7D). These results demonstrate that phototropin-mediated blue light-induced growth enhancement occurs in natural environments at low PAR and that *phot2* as well as *phot1* take part in the response.

DISCUSSION

Phototropins Mediate Growth Enhancement in Response to Blue Light

In this study, we demonstrated that a low intensity of blue light superimposed on red light induced increased growth in *Arabidopsis* green tissue and that the growth enhancement was particularly prominent when the plants were cultivated under low PAR of red light (Figures 2 and 3). The green tissue fresh weight reached threefold values when plants were grown under low PAR if only 0.4% of the PAR was supplemented as blue light. Such a dramatic growth enhancement was probably due to the fact that the PAR chosen was close to the light compensation point for CO₂ fixation. In accordance with this notion, the degree of growth enhancement by blue light was decreased (Figure 3D) when PAR was increased to the moderate level of $70 \mu\text{mol m}^{-2} \text{s}^{-1}$. These findings may become important in the commercial cultivation of vegetable crops.

We have demonstrated that the blue light-dependent growth enhancement was mediated by phototropins. The involvement of other blue light absorbing receptors could be ruled out because the double mutants *phyA phyB* and *cry1 cry2* exhibited clear growth enhancement in response to blue light (Figure 2A). These findings suggested that phototropins functioned to optimize photosynthetic performance. Our attempts to estimate photosynthetic efficiency by monitoring green tissue growth substantiated this interpretation.

phot1 Is More Sensitive to Blue Light in Promoting Plant Growth than *phot2*

We found that both *phot1* and *phot2* took part in the growth enhancement, but the *phot1*-mediated pathway was more sensitive than the *phot2*-mediated one (Figure 3). This may be accounted for by the depression of *PHOT2* expression under our growth conditions because the expression depends on blue light irradiation (Jarillo et al., 2001; Kagawa et al., 2001). However, *phot2* was expressed under red light, and its levels were not increased by additional blue light ($0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$; Figure 4) or by white light ($70 \mu\text{mol m}^{-2} \text{s}^{-1}$; data not shown) in which the blue

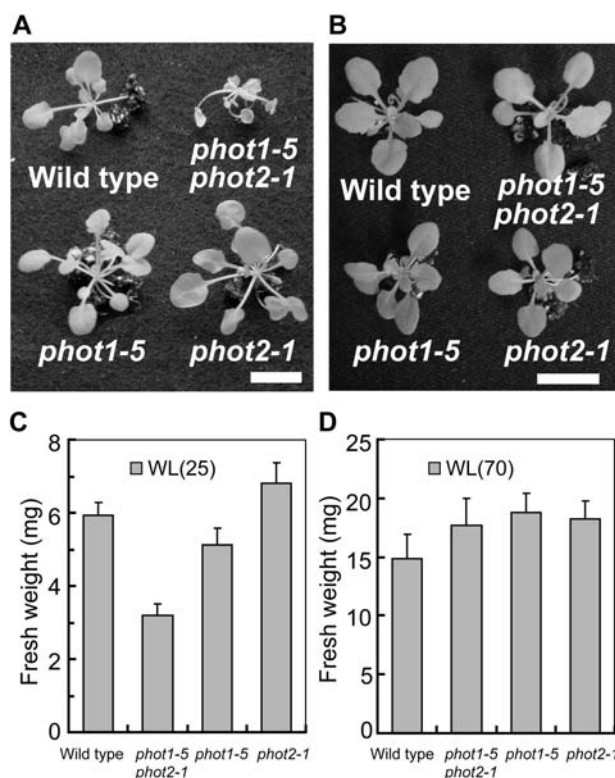


Figure 7. Plants Grown under White Light (12 h Photoperiod).

(A) and (B) Plants of the wild type, *phot1-5*, *phot2-1*, and *phot1-5 phot2-1* grown for 4 weeks at $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light (A) and at $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light (B). Bars = 1 cm.

(C) and (D) Fresh weight of green tissues of the wild type and mutants grown for 4 weeks at $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light (WL) (C) and at $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light (D). Bars represent means \pm SE ($n = 24$).

light intensity was much higher than under standard conditions (Figure 7B). The difference in the sensitivity of plant growth to blue light is in accordance with biochemical properties of phototropins (Salomon et al., 2000; Christie et al., 2002; Kasahara et al., 2002; Chen et al., 2004). A flavin-cysteinyl adduct, which forms between Cys residues in LOV domains and flavin mononucleotide, is produced in the phototropin molecule under blue light. This photoproduct, which in turn leads to activate a kinase, has a longer life time in *phot1* than in *phot2* in the dark (Christie et al., 2002; Harper et al., 2003; Chen et al., 2004). Moreover, it is in line with the recent finding that *phot1* mediates a rise in cytosolic Ca^{2+} in response to $0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$ blue light, whereas the analogous *phot2*-dependent effect requires $>1 \mu\text{mol m}^{-2} \text{s}^{-1}$ blue light (Harada et al., 2003).

Physiological Responses Mediated by Phototropins

The growth response to very low intensity of blue light was surprisingly strong. Under our standard growth conditions, we found chloroplast accumulation at periclinal cell surfaces and increased average stomatal apertures in both the wild type and *phot2*, but not in *phot1* and *phot1 phot2* (Figures 6A and 6B). These blue light responses are likely to increase the efficiency of photosynthetic CO_2 fixation, particularly under low PAR. Therefore, we measured photosynthetic CO_2 fixation and found increased rates in the wild type and *phot2* under standard growth conditions (Figure 6C). Although the enhancement of the photosynthetic CO_2 fixation rate corresponded to only 5% of the maximum rate, it may promote overproportionate growth increments at light intensities close to the compensation point for CO_2 fixation, as in our experiments (Figure 1).

We further found that the surface area of leaves exposed to light was reduced by 50% because of curling in *phot1* and *phot1 phot2* (Figure 6D). Leaf curling may decrease the photosynthetic efficiency by reducing the effective light-capturing leaf area. Interestingly, leaves curled in the wild type as well as in the double mutant if irradiated with low and moderate red light (see supplemental data online).

It would appear possible that the growth enhancement observed is caused by a phototropin-mediated transcriptional regulation of growth-relevant genes. However, recent microarray analyses suggest that phototropins only play a minor role in blue light-controlled transcriptional regulation (Ohgishi et al., 2004).

Role of Phototropins in the Natural Environment

Under normal illumination ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$ white light), no significant differences in growth became evident between the *Arabidopsis* wild type and phototropin mutants (Kasahara et al., 2002; Sakamoto and Briggs, 2002; Figure 7). However, under low PAR, *phot1* mediates a remarkable increase of green tissue fresh weight in response to blue light (Figures 2 and 3). The simultaneous optimization of chloroplast localization and stomatal opening and the maximization of effective leaf area under low PAR (Figure 6) probably contribute to increased photosynthetic rates and, consequently, accelerated growth. Furthermore, a substantial *phot1*-dependent growth enhancement was observed also under moderate PAR (Figure 3D) corresponding to

frequently encountered light intensities in the natural environment (Vogelmann, 2002). Moreover, *phot2* mediated growth responses under light-limited conditions in addition to the *phot1*-dependent effect, when the supplemental blue light intensity was increased (Figure 3C).

In natural environments, phototropism and sun tracking also play an important role in plant growth (Briggs and Christie, 2002), in contrast with our defined experimental conditions where the light source was fixed. The phototropin-mediated responses are integrated to optimize photosynthesis and provide distinct advantages for the survival of plants growing in low-light environments, including dense canopies, dawn, and cloudy daytime, and may have played an important role in higher plant evolution. It is interesting to note the role of fern photoreceptor phytochrome3 (*phy3*), a chimeric protein with a red/far-red-light-absorbing phytochrome and a phototropin (Nozue et al., 1998). *phy3* greatly enhances the sensitivity of phototropism and chloroplast movement to white light in fern *Adiantum* (Kawai et al., 2003) and is likely to play a central role in the divergence and proliferation of polypod fern in a low light environment, which had been made after the proliferation of angiosperms in the Cretaceous period (Schneider et al., 2004). Finally, we note that recent study, using wild-type, *phot1*, *phot2*, and *nph3* mutants, suggested the adaptive function of phototropins in the seedling emergence in the field conditions (Galen et al., 2004).

METHODS

Plant Materials

Arabidopsis thaliana plants of the wild type (ecotype Columbia), *phot1-5* (Huala et al., 1997), *phot2-1* (Kagawa et al., 2001), *phot1-5 phot2-1* (Kagawa et al., 2001), *hy4-3 cry2-1* (ecotype Wassilewskija; Ahmad et al., 1998), and *phyA-201 phyB-5* (ecotype Landsberg; obtained from the ABRC) were grown from seeds at $22 \pm 3^\circ\text{C}$ for 4 to 6 weeks under continuous red light ($25 \pm 4 \mu\text{mol m}^{-2} \text{s}^{-1}$) and blue light ($0.1 \pm 0.01 \mu\text{mol m}^{-2} \text{s}^{-1}$) unless otherwise stated (standard growth conditions). Three transformant lines of homozygous T2 generation plants (A, B, and C; see below) were grown under the same conditions. To determine red light-dependent light-response curves of the CO_2 assimilation rate, rosette leaves of 5- to 6-week-old plants grown under fluorescent light ($55 \mu\text{mol m}^{-2} \text{s}^{-1}$) were used.

Red light and blue light were provided by light-emitting photodiodes (LED-R, maximum intensity at 660 nm, and Stick-B-32, maximum intensity at 470 nm; Eyela, Tokyo, Japan). For the determination of the light-response curves, red light was obtained by passing light from a halogen lamp (MHF-G150-LR; Moritex, Tokyo, Japan) through a red glass filter (Corning 2-61; Corning, NY). White light was obtained from fluorescent lamps (FL 40S N-SDL; National, Tokyo, Japan). Photon flux density was measured using a light meter (LI-250; Li-Cor, Lincoln, NE) equipped with a light sensor (LI-190 SA; Li-Cor).

Generation of Transgenic Arabidopsis

The cDNA encoding full-length *phot1* of *Arabidopsis* was cloned using specific primers (5'-CCGGATCCAAGATGGAACCAACAGAAAAAC-3' and 5'-CCGGATCCCTCAAAAAACATTTGTTTGCAG-3'). The *phot1-5 phot2-1* double mutant was transformed with 35S *Cauliflower mosaic virus* promoter-driven *PHOT1* cDNA, which was inserted into the pBI121 vector through *Agrobacterium tumefaciens* (Doi et al., 2004). Homozygous T2 generations, named A, B, and C, were obtained.

Immunological Analysis of Phototropin Proteins

Microsomal fractions were prepared from rosette leaves of 4-week-old wild-type and phototropin mutants. Proteins of microsomal fractions were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Laemmli, 1970). Immunodetection of phot1 and phot2 was performed with polyclonal antibodies raised against *Arabidopsis* phot1 and phot2, respectively (Doi et al., 2004). phot1 antibodies recognized the protein specifically, whereas phot2 antibodies recognized both phot1 and phot2 (Figure 4). Plasma membrane H⁺-ATPase was quantified immunologically with polyclonal antibodies as described previously (Kinoshita and Shimazaki, 1999). Total protein was determined as described before (Bradford, 1976).

Determination of Growth and Physiological Parameters

For green tissue fresh weight determination, aerial parts of 4-week-old plants were excised and weighed immediately. Chloroplast localization in living mesophyll cells was monitored using micrographs taken from the adaxial sides of detached rosette leaves (Kasahara et al., 2002).

For the measurement of stomatal apertures, rosette leaves were detached and infiltrated immediately with water under vacuum. Stomata on the abaxial sides of the leaves were examined with a microscope (Optiphot; Nikon, Tokyo, Japan). The apertures were expressed as the ratio of stoma width (distance between outer edges of guard cell dorsal walls) and the length of the guard cells.

Leaf surface area was determined in rosette leaves that were detached and photographed. The leaves were uncurled, and photographs were taken again. Leaf areas before and after artificial flattening were compared.

Gas exchange was measured with a portable gas exchange system (LI-6400; Li-Cor) equipped with an *Arabidopsis* leaf chamber (6400-15; Li-Cor). Measurements were done at 24°C, with a reference CO₂ concentration of 350 μmol mol⁻¹ and relative humidity of 45 to 60%. To measure photosynthetic CO₂ fixation under our standard growth conditions, gas exchange characteristics were determined in plants in the growth room. Curled leaves of mutants were flattened artificially before measurement. Chlorophyll was determined as detailed elsewhere (Porra et al., 1989).

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